

Supporting Information

A Panel of TrpB Biocatalysts Derived from Tryptophan Synthase through the Transfer of Mutations that Mimic Allosteric Activation

*Javier Murciano-Calles⁺, David K. Romney⁺, Sabine Brinkmann-Chen, Andrew R. Buller, and Frances H. Arnold**

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Table of Contents

1. Alignment of TrpB homologs.....	S-3
<i>Figure S1.</i> Multiple sequence alignment of TrpB homologs	S-3
<i>Figure S2.</i> Structural alignment of <i>PfTrpB</i> and <i>EcTrpB</i>	S-4
2. Characterization of <i>TmTrpS</i>, <i>TmTrpB</i>, and variants with 0B2 mutations	S-5
<i>Figure S3.</i> k_{cat} values of <i>TmTrpS</i> and all studied variants of <i>TmTrpB</i>	S-5
<i>Figure S4.</i> UV-vis spectra of <i>TmTrpS</i> and all <i>TmTrpB</i> studied variants	S-6
3. Libraries of <i>EcTrpB</i> variants	S-7
<i>Figure S5.</i> (a) S297X <i>EcTrpB</i> library	S-7
<i>Figure S5.</i> (b) 0B2 <i>EcTrpB</i> recombination library	S-7
4. Analysis of <i>PfTrpB</i>^{M144T N166D} and homologous variants	S-8
<i>Figure S6.</i> Frequency of residues present at positions 144 and 166	S-8
<i>Figure S7.</i> UV-vis absorption spectra of the double mutants	S-9
<i>Figure S8.</i> Percent conversion to 5-bromotryptophan attained by several activated TrpB variants	S-9
5. Procedures for enzyme expression and characterization.....	S-10
5.1 Cloning, expression, and purification of TrpB homologs	S-10
5.2 Library construction	S-10
5.3 Library screening.....	S-11
5.4 Kinetics and spectroscopy	S-11
<i>Table S1.</i> Kinetic parameters for all studied enzymes	S-12
6. Synthesis and characterization of tryptophan derivatives.....	S-12
6.1 General information for biocatalytic reactions.....	S-12
6.2 Reactions to compare relative rates.....	S-13
6.3 Estimation of relative rates.....	S-13
<i>Table S2.</i> Comparison of relative rates.....	S-13
6.4 Preparative reactions for product characterization	S-14
6.5 Determination of optical purity	S-14
5-chlorotryptophan (Table 4, entry 1)	S-14
5-bromotryptophan (Table 4, entry 2).....	S-15
5-nitrotryptophan (Table 4, entry 3).....	S-16
5-cyanotryptophan (Table 4, entry 4).....	S-16
5-formyltryptophan (Table 4, entry 5).....	S-16
5-boronotryptophan (Table 4, entry 6)	S-17
5-hydroxytryptophan (Table 4, entry 7)	S-17
5-methyltryptophan (Table 4, entry 8)	S-18
5-methoxytryptophan (Table 4, entry 9)	S-19
7. References.....	S-19
8. ¹H and ¹³C NMR spectra of tryptophan derivatives.....	S-20

1. Alignment of TrpB homologs

OB2 MUTATIONS	L G	
<i>Pyrococcus furiosus</i>	-----MWFGEFGGQYVPETLIEPLKELEKAYKRFKDDDEEFNRQLNYYLKTWA	47
<i>Archaeoglobus fulgidus</i>	MRCWLENLSGGRKMKFGEFGGRFVPEVLIPPLEELEKAYDRFKDDEEFKARLEYLKSVA	60
<i>Thermotoga maritima</i>	-----MKGYFGPYGGQYVPEILMPALEELEAAAYEIMKDESFWKEFNDLLRDYA	49
<i>Escherichia coli</i>	-----MTTLLNPYFGEFGGMYPQILMPALRQLEEFVSAQKDPFQAQFNDLLKNYA	53
	* * : * * : * * : * : * : * * : * : * : * : * : *	
OB2 MUTATIONS	V	
<i>Pyrococcus furiosus</i>	GRPTPLYAKRLTEKIGGAKIYLKREDLVHGGAHKTNNAIGQALLAKFMGKTRLIAETGA	107
<i>Archaeoglobus fulgidus</i>	GRPTPLYFAENLSREL-GVKIYLKREDLLHGGAHKINNTIGQALLAKFMGKKRVIAETGA	119
<i>Thermotoga maritima</i>	GRPTPLYFARRLSEKY-GARIYLKREDLLHTGAHKINNAIGQVLLAKKMKGKTRIIAETGA	108
<i>Escherichia coli</i>	GRPTALTKCQNITAGT-NTTLYLKREDLLHGGAHKTNQVLGQALLAKRMGKTEIIAETGA	112
	**** * . . . : . : ***** : * **** * : : * : * : * : * : * : * : *	
OB2 MUTATIONS		
<i>Pyrococcus furiosus</i>	GQHG VATAMAGALLGMKVDIYMGAE DVERQKMN VFRMKLLGANVIPVNSGSRTLKDAINE	167
<i>Archaeoglobus fulgidus</i>	GQHG VATAMAAALLGLEAEIYMGAE DYERQKMN VFRMELLGAKVTAVESGSRTLKDAINE	179
<i>Thermotoga maritima</i>	GQHG VATATAAALFGMECVIYMGEE DTRQKPNVFRMKLLGAKVVPVKS GSRTLKDAINE	168
<i>Escherichia coli</i>	GQHG VASALASALLGLKCR IYMGAKDVERQSPNVFRMLMGA EVIPVHSGSATLKDACNE	172
	***** : * . * . : : * * : * * . * * * . * : * : * * * * * * * *	
OB2 MUTATIONS		
<i>Pyrococcus furiosus</i>	ALRDWVATFEYTHYLIGSVVGPHPYPTIVRDFQSVIGREAKAQILEAEGQLPDVIVACVG	227
<i>Archaeoglobus fulgidus</i>	ALRDWVESFEHTHYLIGSVVGPHPPTIVRDFQAVIGKEARRQIEAEGMPDAIIACVG	239
<i>Thermotoga maritima</i>	ALRDWITNLQTTYVIGSVVGPHPYPIIVRN FQK VIGEETKKQILEKEGRLPDYIVACVG	228
<i>Escherichia coli</i>	ALRDWSGSYETAHYMLGTAAGPHPYPTIVREFQRMIGEETKAQILEREGRLPDAVIACVG	232
	***** . : : * : : * : : * : * : * : * : * : * : * : * : * : * : * : *	
OB2 MUTATIONS	S	
<i>Pyrococcus furiosus</i>	GGSN AMGIFYPFVNDKKV LKLVGEAGGKLGESGKHSASLNAGQVGVF HGMLSYFLQDEEG	287
<i>Archaeoglobus fulgidus</i>	GGSN AMGIFHPFLND-DVRLIGVEAGGEGIESGRHSASITAGSKGV LHGMLSYFLQDEEG	298
<i>Thermotoga maritima</i>	GGSN AAGIFYPFIDS-GVKLIGVEAGGELETGKHAASLLKKG IGYLHGSKTFVLQDDWG	287
<i>Escherichia coli</i>	GGSN AIGMFADFINETNVGLIGVEPGHG IETGEHGAPLKHGRVGIYFGMKAPMMQTEDG	292
	***** : * * : : . * * : * * * * : * : * . * * * * . * : : * : *	
OB2 MUTATIONS	S A	
<i>Pyrococcus furiosus</i>	QIKPTHSIAPGLDYPGVGPEHAYLKKI QRAEYVTVTDEEALKAFHEL SRTEGIIPALES	347
<i>Archaeoglobus fulgidus</i>	MMLDTHSVSAGLDYPGVGPEHAYLKETGRCEYVTVNDEEALRAFKTL SKLEGIIPALES	358
<i>Thermotoga maritima</i>	QVQVTHSVSAGLDYSGVGPEHAYWRETGKVLYDAVTDEEALDAFIELSRLEGIIPALES	347
<i>Escherichia coli</i>	QIEESYSISAGLDFPSVGPQHAYLNSTGRADYVSITDDEALEAFKTLCLHEGIIPALES	352
	: : : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
OB2 MUTATIONS		
<i>Pyrococcus furiosus</i>	HAVAYAMKLAK-MSRDEIIIVNL SGRGDKDLDIVLKVSGNV----	388
<i>Archaeoglobus fulgidus</i>	HAIAYAMKMAEE-MQRDDVLVVNL SGRGDKDMDIVRRRLA-----	397
<i>Thermotoga maritima</i>	HALAYLKIN---IKGKVVVVNL SGRGDKDLESVLNHPYVREIR	389
<i>Escherichia coli</i>	HALAHALKMMRENPDKEQLLVVNL SGRGDKDIFTVHDILKARGEI-	397
	* : * : * : : : : * : : * : * : * : * : *	

Figure S1. Multiple sequence alignment of *Pyrococcus furiosus*, *Archaeoglobus fulgidus*, *Thermotoga maritima*, and *Escherichia coli* TrpB homologs. The mutated residues in *Pf*TrpB^{OB2} are shown in red in the first line. Active site residues (distance to active site < 3.2Å) are colored in blue whereas those involved in proton transfer during catalysis are colored in green. Symbols under sequences signify: (*) identical residues, (:) residues of equal nature, and (.) similar residues.

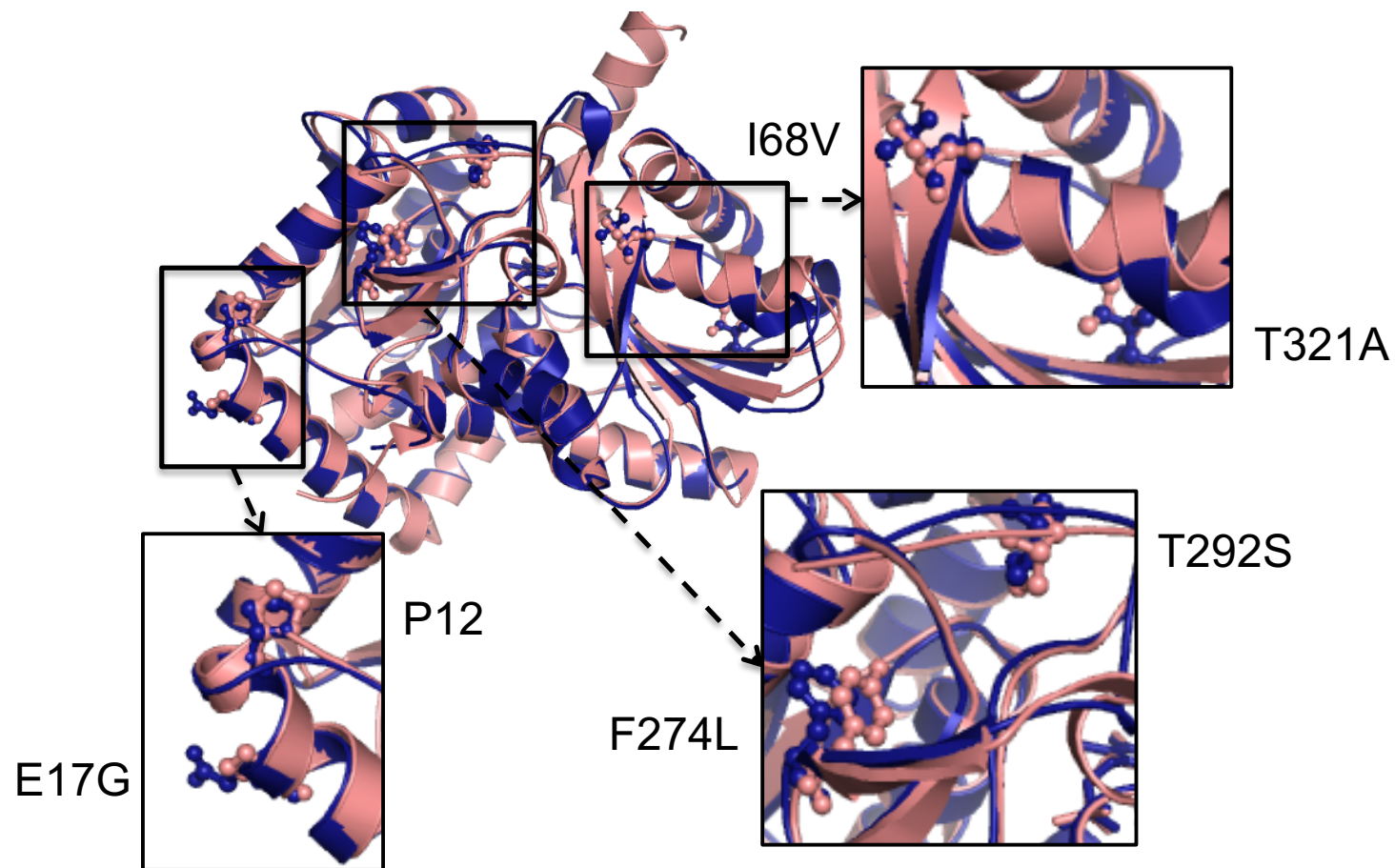


Figure S2. Structural alignment of *Pf*TrpB (PDB entry 5DVZ) and *Ec*TrpB (PDB entry 2DH5). The residues mutated in *Pf*TrpB^{OB2} are shown in sticks. Windows show the region surrounding each labeled mutation

2. Characterization of *Tm*TrpS, *Tm*TrpB, and variants with 0B2 mutations

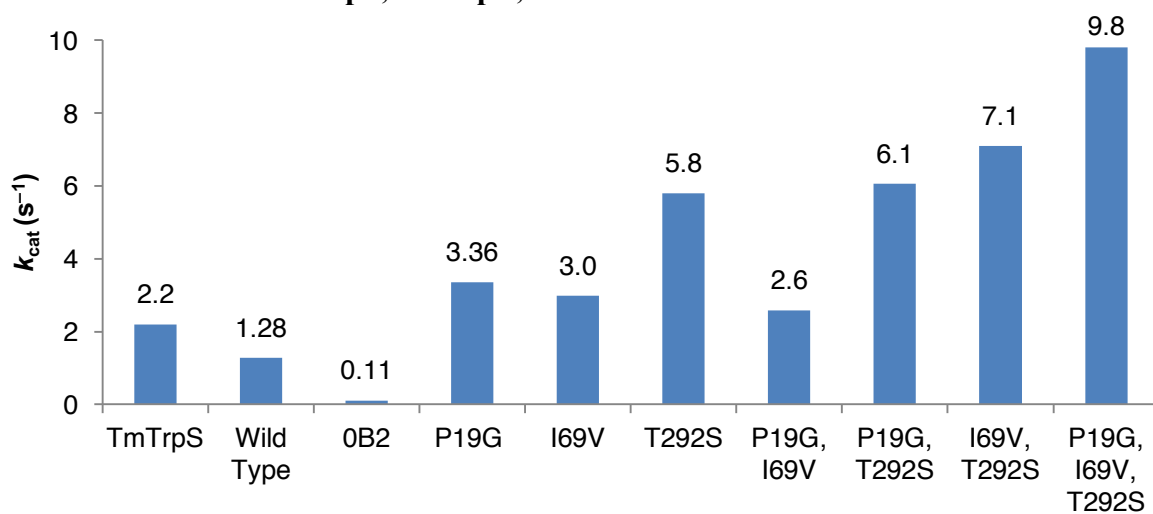


Figure S3. k_{cat} values of *Tm*TrpS and all studied variants of *Tm*TrpB. The mutations are labeled below each bar. Measurements were performed using the procedure described in section 5.4. The standard error of the fit is <20%.

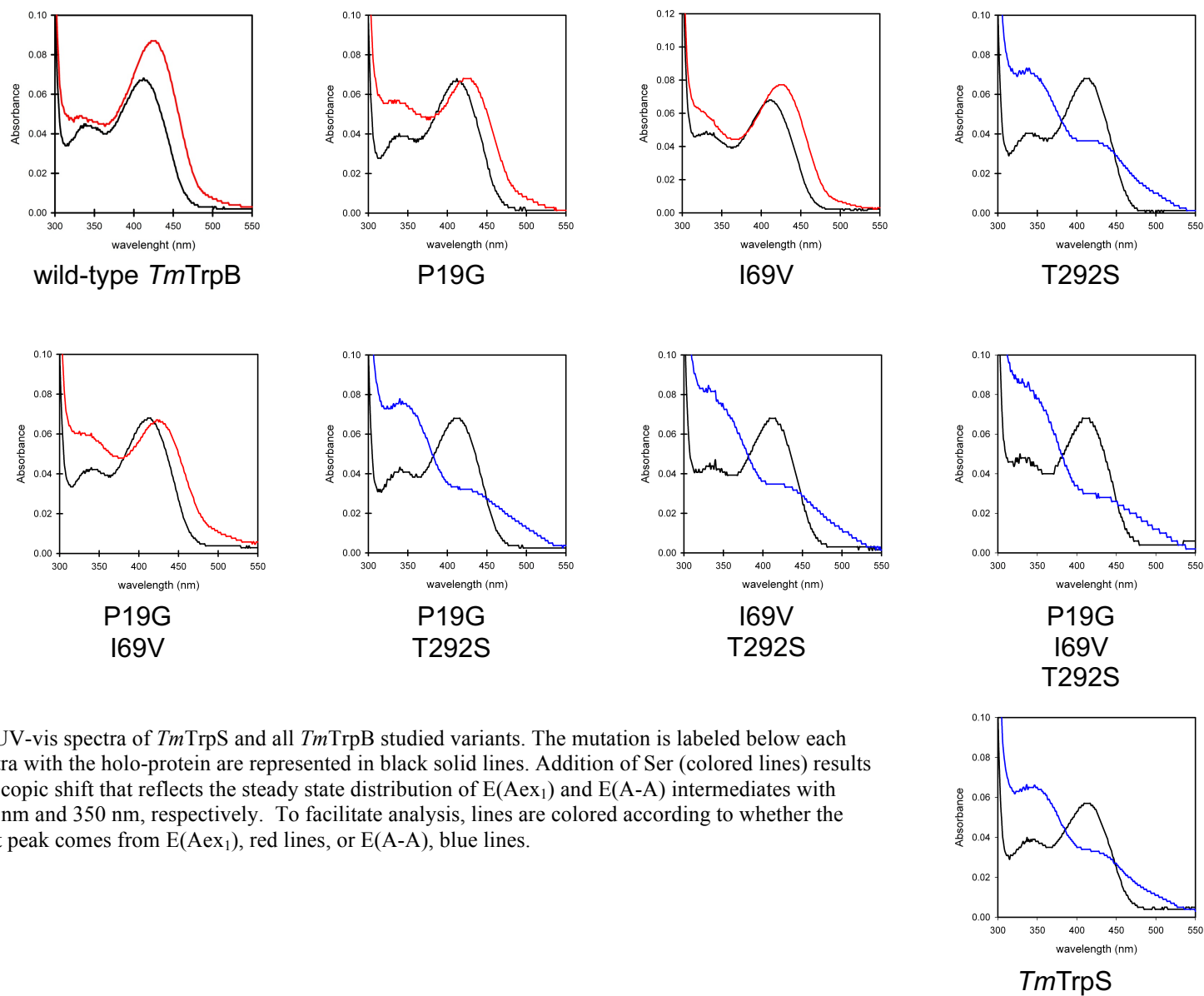


Figure S4. UV-vis spectra of *TmTrpS* and all *TmTrpB* studied variants. The mutation is labeled below each graph. Spectra with the holo-protein are represented in black solid lines. Addition of Ser (colored lines) results in a spectroscopic shift that reflects the steady state distribution of E(Aex₁) and E(A-A) intermediates with $\lambda_{\text{max}} = 428 \text{ nm}$ and 350 nm , respectively. To facilitate analysis, lines are colored according to whether the predominant peak comes from E(Aex₁), red lines, or E(A-A), blue lines.

3. Libraries of *Ec*TrpB variants

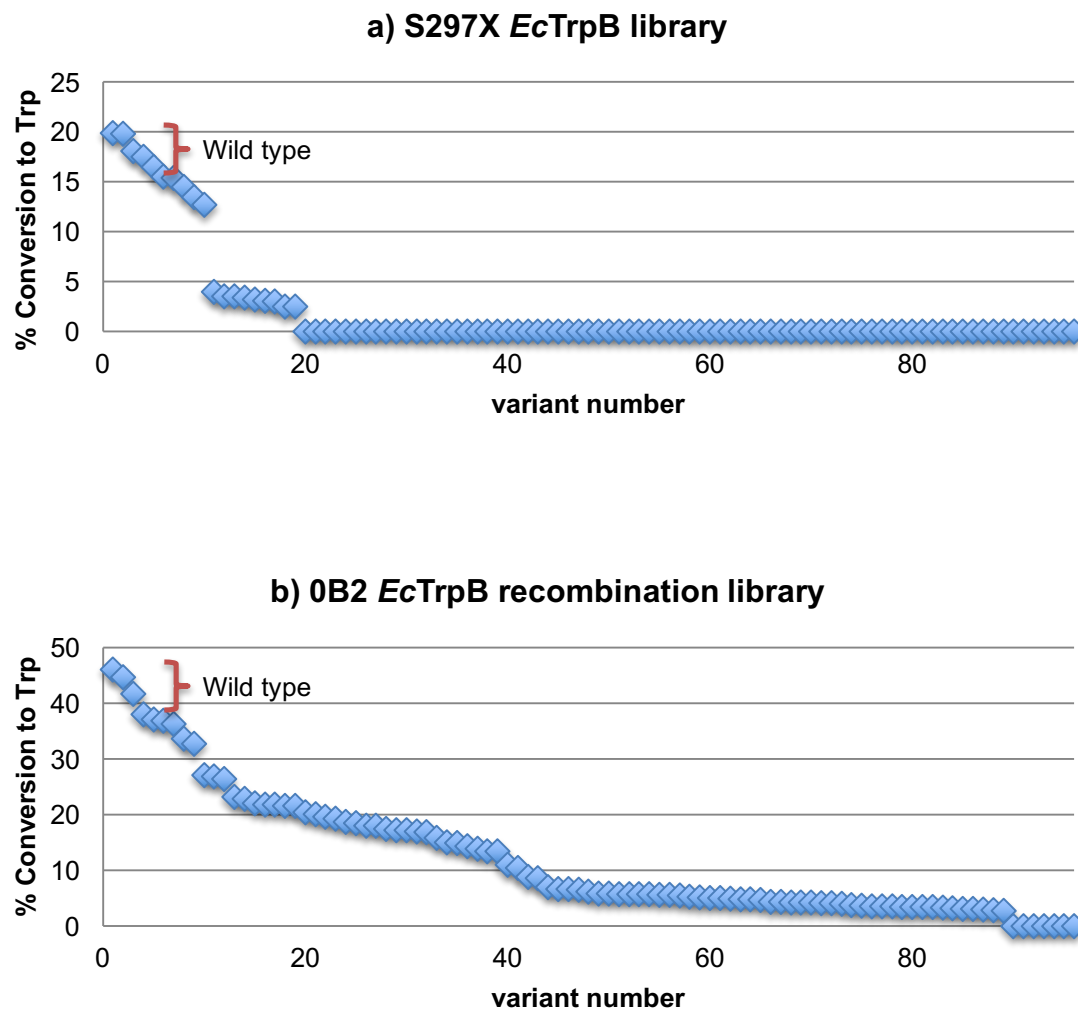


Figure S5. a) Activity profile of a site-saturation library at residue S297 in *Ec*TrpB. b) Activity profile of a recombination library of the 0B2 mutations (see methods for details).

4. Analysis of *Pf*TrpB^{M144T N166D} and homologous variants

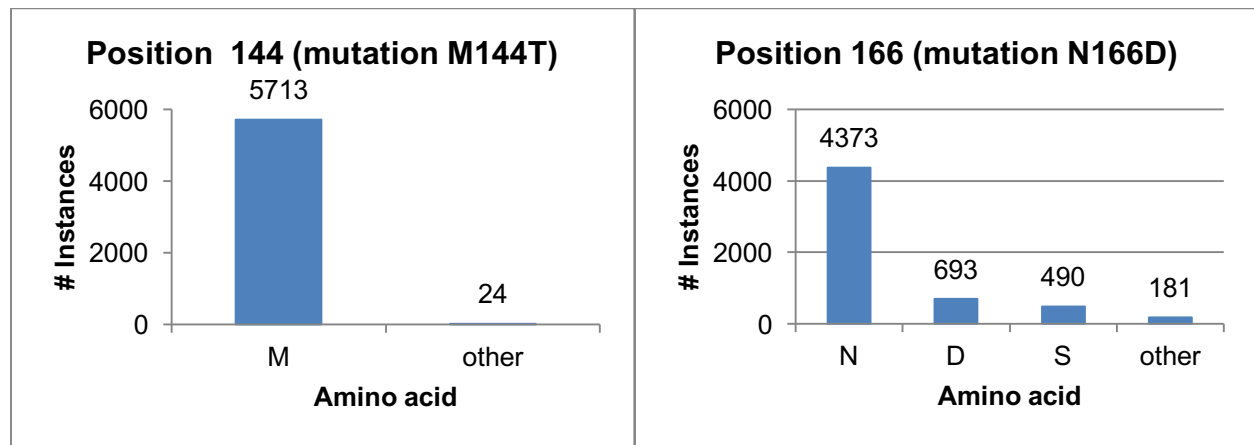


Figure S6. Frequency of residues present at positions 144 and 166 (according to *Pf*TrpB numeration) across different species.

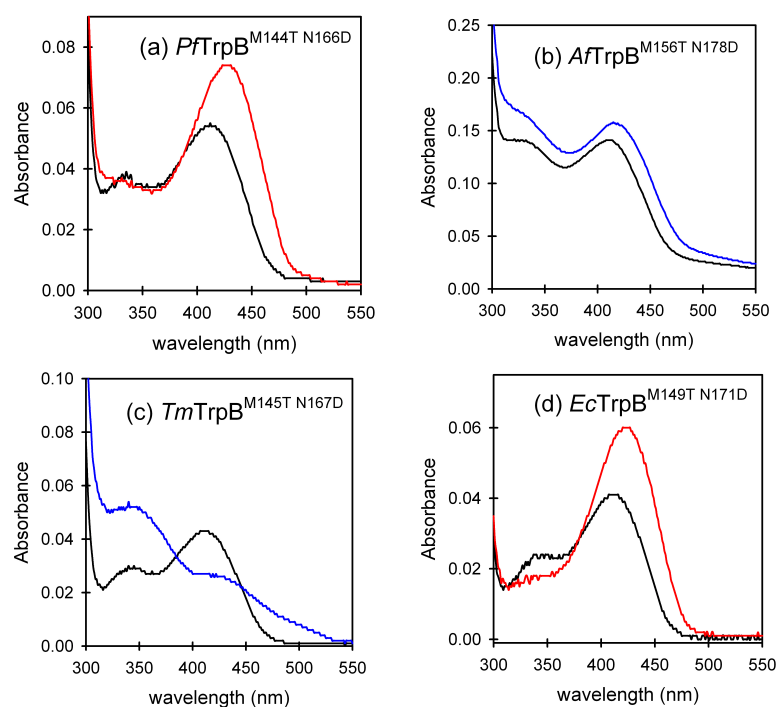


Figure S7. UV-vis absorption spectra of the double mutants of (a) *P. furiosus*, (b) *A. fulgidus*, (c) *T. maritima*, and (d) *E. coli*. Spectra with the holo-protein are represented in black solid lines. Similar to Figure S4, recorded spectra after addition of L-serine are represented in blue solid lines when the predominant absorbance peak belongs to E(A-A), and red when it belongs to E(Aex₁).

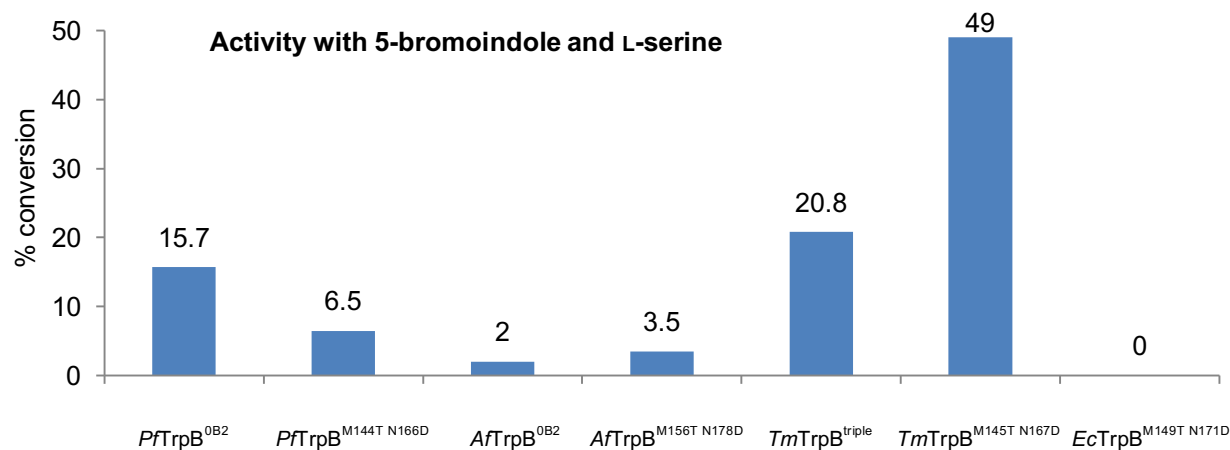


Figure S8. Percent conversion to 5-bromotryptophan attained by several activated TrpB variants. Reactions with *Pf* and *Tm* variants were done at 75 °C, whereas reactions with *Af* and *Ec* variants were done at 37 °C. Reaction conditions are identical to those reported for 5-bromoindole in Section 6.2, except the reactions were run for 12 hours. Conversion was determined by LCMS analysis.

5. Procedures for enzyme expression and characterization

5.1 Cloning, expression, and purification of TrpB homologs

The genes encoding *Af*TrpB (UNIPROT ID O28672), *Tm*TrpB (UNIPROT ID P50909), and *Ec*TrpB (UNIPROT ID P0A879) were obtained from Integrated DNA Technologies (IDT, San Diego, USA) as gBlocks and cloned into pET22(b)+ using Gibson assembly.^[1] Protein expression of the homologs was carried out in *Escherichia coli* BL21 *E. coli* Express cells (Lucigen) by inoculating 10 mL Terrific Broth containing 100 µg/mL ampicillin (TB_{amp}) with a single colony and incubating this pre-culture over night at 37 °C and 250 rpm. The overnight cultures were used to inoculate 500-mL TB_{amp} expression cultures to an OD₆₀₀ of 0.1. After shaking at 250 rpm and 37 °C for ~3 h, the cultures were chilled on ice for 20 min and then induced with IPTG to a final concentration of 1 mM. Expression of the homologs took place at 250 rpm and 20 °C for another 20 h. After centrifugation, the cell pellets were frozen and stored at –20 °C until further use.

For protein purification, cells were thawed and then resuspended in 50 mM potassium phosphate buffer (KPi), pH 8.0, containing 20 mM imidazole, 100 mM NaCl (buffer A), and 200 µM PLP. Lysis was performed with BugBuster (Novagen) for 15 min at 37 °C. After removal of the cell debris by centrifugation, *Tm*TrpB (but not *Af*TrpB or *Ec*TrpB) lysate was then incubated at 75 °C for 10 min and was subjected to another centrifugation step. Afterwards, purification was done using a 1-mL histrap HP column with an AKTA purifier FPLC system (GE Healthcare) and a linear gradient from buffer A to buffer B (50 mM KPi, pH 8.0, 500 mM imidazole, and 100 mM NaCl). Proteins eluted at approximately 140 mM imidazole. Purified proteins were dialyzed into 50 mM KPi, pH 8.0, flash-frozen in liquid N₂, and stored at –80 °C until further use.

The *Tm*TrpA and *Af*TrpA genes were also obtained from IDT as gBlocks and cloned into pET28 via Gibson assembly using restriction enzymes *Nco*I and *Xho*I on the backbone. To generate the complexes *Tm*TrpS or *Af*TrpS, both the A and the B subunit of each homolog were independently expressed and purified as described above. Afterwards, purified proteins were mixed using a final ratio of 1:3 (TrpB:TrpA).

Protein concentrations were determined via the Bradford assay (Bio-Rad).

5.2 Library construction

For *Tm*TrpB, a recombination library of mutations P14L, P19G, I69V, L274S, and T292S was constructed using site-directed mutagenesis by overlap extension (SOE) PCR.^[2] In this library, we allowed both the native residue and the mutation at each of the sites. After generating the fragments via PCR, they were *Dpn*I digested, gel purified, and used as template for the subsequent assembly PCR using the flanking primers only. The assembly PCR product was cloned via Gibson into pET22(b)+ between restriction sites *Nde*I and *Xho*I.

For the *Ec*TrpB recombination library, the process was analogous and the recombined mutations were P18G, P23G, L73V, Y279S, and S326A. For the *Ec*TrpB site-saturation library at position S297, three primers were designed containing codons NDT (encoding for Ile, Asn, Ser, Gly, Asp, Val, Arg, His, Leu, Phe, Tyr, and Cys), VHG (encoding for Met, Thr, Lys, Glu, Ala, Val, Gln, Pro, and Leu), and TGG (Trp), respectively, thereby including all 20 natural amino acids. These three primers were mixed in a ratio 12:9:1 according to the 22-codon-trick.^[3] Then, the SOE PCR was performed as described above.

5.3 Library screening

For *Tm*TrpB library screening, BL21 E. coli Express cells carrying *Tm*TrpB wild-type and variant plasmids were grown in 96-well deep-well plates in 300 μ L/well TB_{amp} at 37 °C and 80% humidity with shaking at 250 rpm overnight. Then, 20 μ L of the overnight cultures were transferred to 630 μ L TB_{amp} and allowed to grow at 37 °C and 80% humidity with shaking at 250 rpm for 3 h. After chilling the cultures on ice for 20 min, IPTG was added to a final concentration of 1 mM. Expression was allowed to continue for another 20 h at 20 °C with shaking at 250 rpm. Cells were then centrifuged at 4,000g for 10 min and frozen at –20 °C for a minimum of 24 h. For screening, cells were thawed at room temperature and then subjected to lysis by the addition of 400 μ L/well of 200 mM KPi buffer, pH 8.0, with 1 mg/mL lysozyme and 0.05 mg/mL DNaseI for 1 h at 37 °C. After centrifugation at 5,000g for 20 min, a 160- μ L aliquot of the lysate was transferred into PCR plates (USA Scientific, Ocala, USA), heat-treated for 1 h at 75 °C, and then spun again at 1,000g and 4 °C for 30 min. After the transfer of 40 μ L of the lysates to a fresh 96-well deep well plate, 160 μ L of assay buffer (200 mM phosphate buffer, 200 μ M indole, 100 mM L-serine, pH 8.0) were added. Reaction was allowed for 15 min at 75 °C and quenched by addition of 200 μ L of CH₃CN. The plates were then centrifuged for 10 min at 4,000g, and 100 μ L of the supernatant were transferred to a 96-well plate for HPLC analysis (4.6 \times 50 mm C-18 silica column with acetonitrile/water: 0% acetonitrile for 1 min, 0% to 100% over 5 min, 100% for 1 min). The conversion was approximated by comparing the integrations of the tryptophan signal (2.4 min) and the indole signal (4.4 min) at 280 nm.

For screening of the *Ec*TrpB library, expression was done analogously to the *Tm*TrpB library; however, we omitted the final freezing step at –20 °C. Instead, once the supernatant was discarded, the pelleted cells were resuspended in 400 μ L/well of 200 mM KPi, pH 8, and 40 μ L were transferred to a fresh 96-well deep-well plate containing 160 μ L of assay buffer (200 mM phosphate buffer, 8 mM indole, 100 mM L-serine, pH 8). After a 1-h reaction at 37 °C, 300 μ L of CH₃CN (83% v/v) were added to stop the reaction. Plates were centrifuged for 10 min at 4,000g. Subsequently, 100 μ L of the supernatant were transferred to a 96-well plate for HPLC, where the ratio between indole and tryptophan was measured as described above for the *Tm*TrpB library.

5.4 Kinetics and spectroscopy

For all TrpB and TrpS enzymes k_{cat} values were measured by recording the change of absorption at 290 nm, using $\Delta\epsilon_{290} = 1.89 \text{ mM}^{-1} \text{ cm}^{-1}$ as determined elsewhere.^[4] Measurements were made in a UV1800 Shimadzu spectrophotometer at 60 °C for *Af*TrpB, 75 °C for *Tm*TrpB, and 37 °C for *Ec*TrpB over 90 seconds. The assay buffer was 200 mM KPi, pH 8, with 40 μ M PLP. Michaelis-Menten constants (K_M) for indole were determined using a concentration range from 400–6.25 μ M indole with a fixed concentration of L-serine of 300 mM. Data fitting was done with the SigmaPlot2000 software.

Spectra were recorded on the UV1800 Shimadzu spectrophotometer using a wavelength range from 250 to 550 nm. The buffer contained 200 mM KPi, pH 8.0, and the protein concentration was 20 μ M. Measurement temperatures were the same as used in the kinetics experiments described above. After a 3-min incubation at the corresponding temperature, the spectrum of the holo-protein was recorded. Then, a 20-mM solution of L-serine was

added and immediately spectra were measured to limit the deamination of L-serine, a side reaction that gives rise to pyruvate,^[5] which absorbs at 320 nm.

Table S1. Kinetic parameters for all studied enzymes.^[a]

Entry	Enzyme	k_{cat} (s ⁻¹)	K_{M} (μM indole)	Entry	Enzyme	k_{cat} (s ⁻¹)	K_{M} (μM indole)
1	<i>Pf</i> TrpB ^{WT}	0.31 ± 0.02	77 ± 12	8	<i>Tm</i> TrpB ^{T292S}	5.8 ± 0.2	25 ± 4
2	<i>Pf</i> TrpB ^{OB2}	2.9 ± 0.2	9 ± 2	9	<i>Tm</i> TrpS	2.2 ± 0.1	44 ± 7
3	<i>Af</i> TrpB ^{WT}	0.074 ± 0.001	12 ± 1	10	<i>Pf</i> TrpB ^{M144T N166D}	0.83 ± 0.03	42 ± 5
4	<i>Af</i> TrpB ^{OB2}	0.51 ± 0.01	4.8 ± 0.6	11	<i>Ec</i> TrpB ^{M149T N171D}	0.34 ± 0.01	18 ± 3
5	<i>Tm</i> TrpB ^{WT}	1.28 ± 0.07	33 ± 7	12	<i>Tm</i> TrpB ^{M145T N167D}	3.3 ± 0.1	32 ± 4
6	<i>Tm</i> TrpB ^{OB2}	0.11 ± 0.02	72 ± 4	13	<i>Af</i> TrpB ^{M156T N178D}	0.34 ± 0.01	11 ± 2
7	<i>Tm</i> TrpB ^{triple}	9.8 ± 0.3	26 ± 3	14	<i>Ec</i> TrpB ^{WI}	0.16 ± 0.01	19 ± 1

[a] Measurement temperatures were 75 °C for *Pf* and *Tm*TrpB, 65 °C for *Af*TrpB, and 37 °C for *Ec*TrpB.

6. Synthesis and characterization of tryptophan derivatives

6.1 General information for biocatalytic reactions

Proton NMR spectra were recorded on a Varian 500 MHz spectrometer. Proton chemical shifts are reported in ppm (δ) relative to tetramethylsilane and calibrated using the residual solvent resonance (D₂O, δ 4.79 ppm). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), doublet of doublets (dd), doublet of doublets of doublets (ddd), doublet of triplets (dt), triplet (t), multiplet (m)], coupling constants [Hz], integration). Carbon NMR spectra were recorded with complete proton decoupling on a Varian 500 MHz (125 MHz) spectrometer or Bruker 400 MHz (100 MHz) spectrometer equipped with a cryo probe. Carbon chemical shifts are reported in ppm relative to tetramethylsilane and calibrated using the residual solvent proton resonance (*vide supra*). All NMR spectra were recorded at ambient temperature (about 25 °C). Reversed-phase chromatography was performed on a Biotage Isolera One purification system, using C-18 silica as the stationary phase, with methanol as the strong solvent and water as the weak solvent. The gradient of the eluent (∇) is given as % strong solvent/column volume (CV). Liquid chromatography/mass spectrometry (LCMS) was performed on an Agilent 1290 UPLC-LCMS equipped with a 2.1 × 50 mm C-18 silica column, using acetonitrile as the strong solvent and 0.1% (v/v) acetic acid/water as the weak solvent. The samples were ionized by electrospray ionization (ESI). High-resolution mass spectrometry (HRMS) was conducted with a JMS-600H (JEOL) instrument, with samples ionized by fast atom bombardment (FAB), or by time-of-flight mass spectroscopy with a Waters LCT Premier XE with UPLC, with samples ionized by ESI. All starting materials were purchased from commercial sources and used without further purification. The optical purity of the products was determined by derivatization with *N*-(5-fluoro-2,4-dinitrophenyl)alanamide (FDNP-alanamide)^[6] as described below.

6.2 Reactions to compare relative rates

The reactions using the 5-chloro, 5-bromo, 5-cyano, 5-hydroxy, 5-methyl, and 5-methoxy substrates used the following procedure: an HPLC vial was charged with 10 μL of a 200 mM stock solution of the indole derivative in DMSO (10 mM final concentration), followed by 174 μL of a stock solution formed by mixing 1730 μL of aqueous potassium phosphate buffer (200 mM phosphate, pH 8.0), 10 μL of aqueous L-serine (2 M, 10 mM final concentration), and 2.7 μL of aqueous PLP (1.5 mM, 2 μM final concentration). The vials were sealed, then placed in a heating block that had been equilibrated to 75 $^{\circ}\text{C}$. After 1 minute, the enzyme (either *Tm*TrpB^{M145T N167D} or *Pf*TrpB^{OB2}) was added as 16 μL of a 5 μM solution in aqueous phosphate buffer (200 mM phosphate, pH 8.0). The reactions were kept at 75 $^{\circ}\text{C}$ for 15 minutes, then cooled in ice. After the reactions were diluted by the addition of 800 μL of 1:1 acetonitrile/water, they were subjected to centrifugation at 20,000g for 10 min and at 4 $^{\circ}\text{C}$. The supernatants were analyzed by LCMS (*vide infra*). All reactions were run in duplicate.

The reactions using the 5-nitro, 5-borono, and 5-formyl substrates were tested using an identical procedure, except for the following modifications: after the indole derivative was added, the vial was charged with 182 μL of a stock solution formed by mixing 1,800 μL of aqueous potassium phosphate buffer (200 mM phosphate, pH 8.0), 10 μL of aqueous L-serine (2 M, 10 mM final concentration), and 13 μL of aqueous PLP (1.5 mM, 10 μM final concentration). The enzyme (either *Tm*TrpB^{M145T N167D} or *Pf*TrpB^{OB2}) was added as 8 μL of a 50 μM solution in aqueous phosphate buffer (200 mM phosphate, pH 8.0).

6.3 Estimation of relative rates

All reaction mixtures, except those of 6-hydroxyindole, were analyzed by LCMS (1 μL injection volume) using a solvent gradient that ranged from 5% to 95% strong solvent over 4 minutes, with a flow rate of 1.0 mL/min. The reaction mixtures generated from 6-hydroxyindole as a substrate were analyzed by a different solvent gradient that ranged from 0% to 20% strong solvent over 4 minutes, also with a flow rate of 1.0 mL/min. The total ion count (TIC) was then filtered for the $[\text{M}+\text{H}]^{+}$ of the expected product. The filtered TICs were integrated and the ratio of the integrations obtained with *Tm*TrpB^{M145T N167D} to those obtained with *Pf*TrpB^{OB2} was taken as an approximation of the relative initial rates for both enzymes.

Table S2. Comparison of relative rates.

X	Integration of TIC		X	Integration of TIC	
	<i>Pf</i> TrpB ^{OB2}	<i>Tm</i> TrpB ^{M145T N167D}		<i>Pf</i> TrpB ^{OB2}	<i>Tm</i> TrpB ^{M145T N167D}
Cl	$(4.0 \pm 0.2) \times 10^5$	$(1.2 \pm 0.1) \times 10^6$	B(OH) ₂ ^[a]	$(2.37 \pm 0.02) \times 10^2$	$(4.28 \pm 0.09) \times 10^2$
Br	$(1.78 \pm 0.03) \times 10^4$	$(1.00 \pm 0.04) \times 10^5$	OH	$(4.09 \pm 0.09) \times 10^5$	$(5.9 \pm 0.2) \times 10^5$
NO ₂	$(4.2 \pm 0.3) \times 10^4$	$(3.1 \pm 0.1) \times 10^5$	CH ₃	$(5.7 \pm 0.2) \times 10^5$	$(8.2 \pm 0.8) \times 10^5$
CN	$(7 \pm 1) \times 10^3$	$(3.07 \pm 0.04) \times 10^4$	OCH ₃	$(2.33 \pm 0.03) \times 10^5$	$(3.52 \pm 0.09) \times 10^5$
CHO	$(1.37 \pm 0.01) \times 10^5$	$(2.57 \pm 0.08) \times 10^5$			

[a] Integration of absorption at 280 nm.

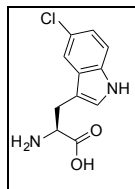
6.4 Preparative reactions for product characterization

General procedure: the indole derivative was weighed directly into a 20-mL reaction vial, then dissolved in DMSO (5% of final reaction volume). Next, L-serine (1.2 or 2 equiv) was added as a 2 M aqueous solution, followed by PLP (5 equiv with respect to enzyme), which was added as a 15 mM aqueous solution. Finally, the reaction mixture was diluted with the appropriate quantity (*vide infra*) of aqueous potassium phosphate buffer (200 mM phosphate, pH 8). The vial was sealed, then placed in a water bath that had been equilibrated to 75 °C. After 1 min, the enzyme *Tm*TrpB^{M145T N167D} (0.01 or 0.02 mol %) was added as a 200-μM solution in aqueous potassium phosphate buffer (50 mM phosphate, pH 8). The reaction was kept at 75 °C for the indicated reaction time (2 or 12 h), then cooled in ice.

The reaction mixture was acidified by the addition of conc. aq. HCl (200 μL), then washed with ethyl acetate (50 mL). The organic phase was washed with 0.1 M aq. HCl (2 × 20 mL), then the combined aqueous layers were concentrated *in vacuo*. The residue was re-dissolved in water, then loaded onto a C-18 column (30 g) that had been equilibrated to 1% methanol/water. The column was washed with five column volumes (CV) of 1% methanol/water to remove residual salts and DMSO. Finally, the product was eluted with a gradient that went from 1% to 100% methanol/water over 10 CV. The fractions containing product were concentrated *in vacuo*. As the free base, the products had poor solubility in most solvents. Thus, they were re-dissolved in 0.1 M aq. HCl. The resulting solutions were partially concentrated on a rotary evaporator, then frozen and concentrated to dryness on a lyophilizer. The products were obtained as the hydrochloride salts, which tended to have good solubility in water.

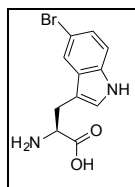
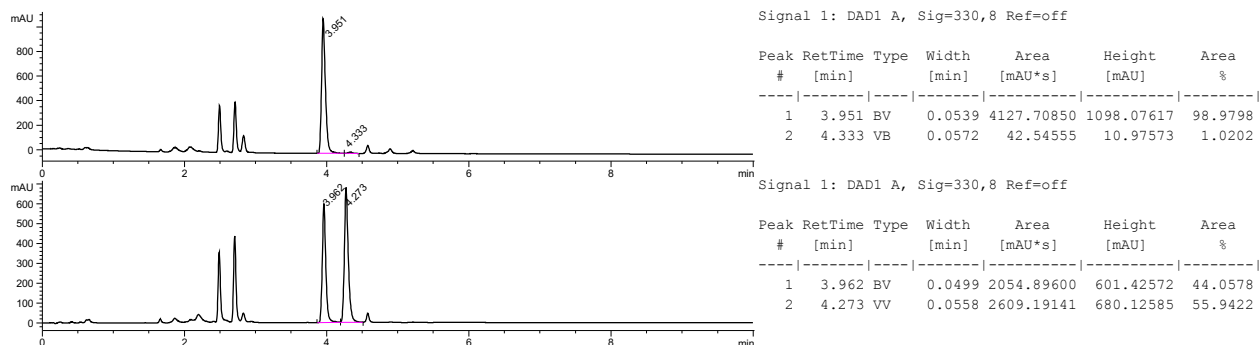
6.5 Determination of optical purity

FDNP-alanamide was used as a solution in acetone (33 mM). In a 2-mL vial, the amino acid (0.50 μmol) was dissolved in 1 M aq. NaHCO₃ (140 μL). FDNP-alanamide (30 μL, 1 μmol) was added, then the vial was placed in an incubator at 37 °C. After 1 h, the reaction mixture was allowed to cool to room temperature, then diluted with 1:1 water/acetonitrile (550 μL). The resulting solution was analyzed directly by LCMS (5 to 95% acetonitrile over 10 minutes, monitoring at 330 nm or using the total ion count filtered for the expected mass. Each amino acid was derivatized with both racemic and enantiopure FDNP-alanamide for comparison. Since the products of derivatization are diastereomeric, they may have different absorption properties and ionization efficiencies. Indeed, the racemic controls indicate that, in all cases, the derivatized minor enantiomer produces stronger signal than the derivatized major enantiomer. Thus, this analysis represents a lower limit on the enantiopurity of the tryptophan derivatives, all of which had >98% ee. Absolute stereochemistry was inferred by analogy to L-tryptophan.

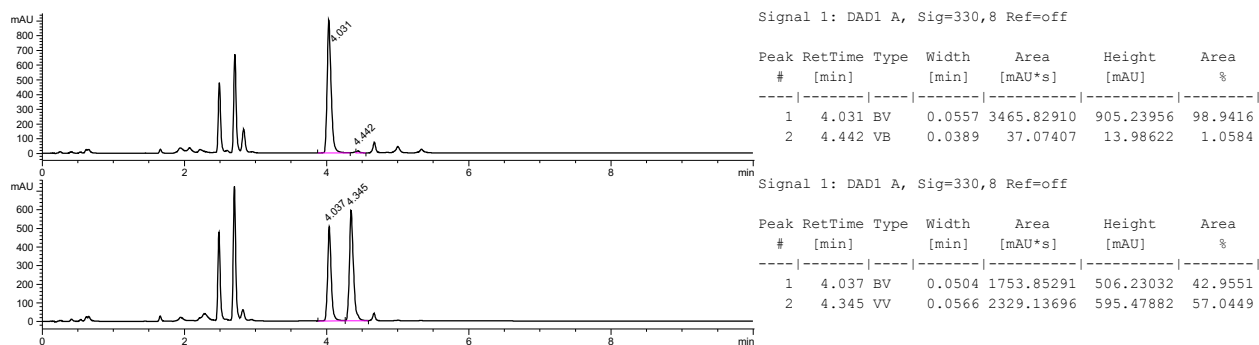


5-chlorotryptophan (Table 4, entry 1). The reaction was performed with 75.8 mg of 5-chloroindole (500 μM), 2 equiv of L-Ser, and 0.01 mol % of enzyme. The starting materials were dissolved in 500 μL DMSO and 8838 μL of potassium phosphate buffer, for a final reaction volume of about 10 mL. The reaction was heated for 12 hours, then processed as described above to afford 5-chlorotryptophan hydrochloride as a white solid (128 mg, 93% yield), corresponding to 9300 turnovers.

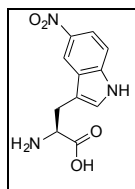
¹H NMR (500 MHz, D₂O) δ 7.63 (d, *J* = 2.0 Hz, 1H), 7.43 (d, *J* = 8.7 Hz, 1H), 7.31 (s, 1H), 7.20 (dd, *J* = 8.7, 2.0 Hz, 1H), 4.32 (dd, *J* = 7.1, 5.3 Hz, 1H), 3.40 (ABX, *J*_{AX} = 7.2 Hz, *J*_{BX} = 5.4 Hz, *J*_{AB} = 15.5 Hz, *v*_{AB} = 33.9 Hz, 2H). **¹³C NMR** (100 MHz, D₂O) δ 171.9, 134.7, 127.6, 126.7, 124.6, 122.1, 117.5, 113.1, 106.1, 53.3, 25.7. **HRMS** (FAB) (*m/z*) for [M+H]⁺ C₁₁H₁₂ClN₂O₂ requires 239.0587, observed 239.0596.



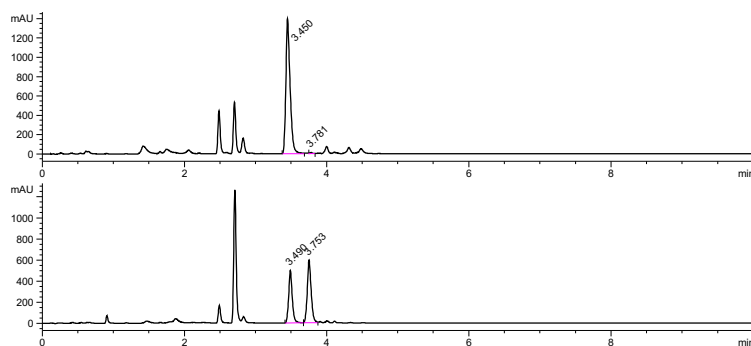
5-bromotryptophan (Table 4, entry 2). The reaction was performed with 19.6 mg (100 μM) of 5-bromoindole derivative, 2 equiv of L-Ser, and 0.02 mol % of enzyme. The starting materials were dissolved in 500 μL DMSO and 9.3 mL of potassium phosphate buffer, for a final reaction volume of about 10 mL. The reaction was heated for 12 hours, then processed as described above to afford 5-bromotryptophan hydrochloride as a white solid (28 mg, 88% yield), corresponding to 4400 turnovers. **¹H NMR** (500 MHz, D₂O) δ 7.75 (s, 1H), 7.36 (d, *J* = 8.6 Hz, 1H), 7.30 (d, *J* = 8.7 Hz, 1H), 7.27 (s, 1H), 4.30 (dd, *J* = 6.7, 5.8 Hz, 1H), 3.36 (ABX, *J*_{AX} = 7.2 Hz, *J*_{BX} = 5.3 Hz, *J*_{AB} = 15.4 Hz, *v*_{AB} = 34.9 Hz, 2H). **¹³C NMR** (100 MHz, D₂O) δ 171.8, 135.0, 128.2, 126.6, 124.7, 120.6, 113.6, 112.1, 106.0, 53.2, 25.6. **HRMS** (FAB) (*m/z*) for [M+H]⁺ C₁₁H₁₂BrN₂O₂ requires 283.0082, observed 283.0094.



Procedure for Table 4, entries 3–5. The reaction was performed with 250 μM of indole derivative, 2 equiv of L-Ser, and 0.02 mol % of enzyme. The starting materials were dissolved in 1250 μL DMSO and 23 mL of potassium phosphate buffer, for a final reaction volume of about 25 mL. The reaction was heated for 12 hours, then processed as described above to afford the tryptophan derivative as the hydrochloride salt.



5-nitrotryptophan (Table 4, entry 3). Yellow solid (18 mg, 25% yield), corresponding to 1250 turnovers. ^1H NMR (500 MHz, D_2O) δ 8.33 (d, J = 2.2 Hz, 1H), 7.87 (dd, J = 9.0, 2.3 Hz, 1H), 7.35 (d, J = 9.2 Hz, 1H), 7.33 (s, 1H), 4.28 (dd, J = 6.9, 5.6 Hz, 1H), 3.36 (ABX, J_{AX} = 6.9 Hz, J_{BX} = 5.5 Hz, J_{AB} = 15.5 Hz, ν_{AB} = 25.2 Hz, 2H). ^{13}C NMR (100 MHz, D_2O) δ 172.0, 140.5, 139.7, 128.4, 125.8, 117.4, 115.76, 111.8, 109.2, 53.4, 25.5. HRMS (FAB) (m/z) for $[\text{M}+\text{H}]^+$ $\text{C}_{11}\text{H}_{12}\text{N}_3\text{O}_4$ requires 250.0828, observed 250.0831.

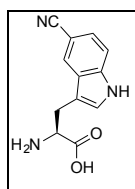


Signal 1: DAD1 A, Sig=330,8 Ref=off

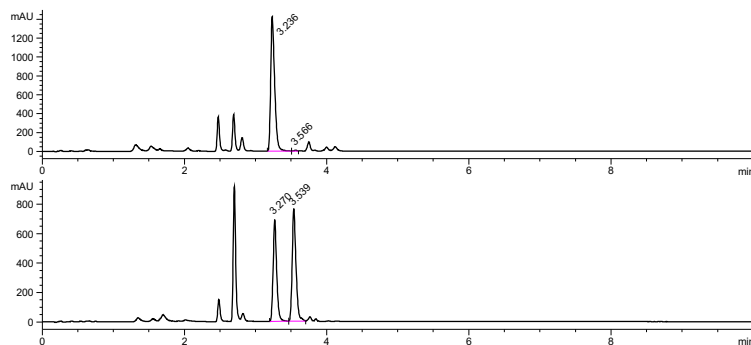
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.450	BV	0.0602	5759.50977	1395.42200	99.5557
2	3.781	VB	0.0391	25.70636	9.96104	0.4443

Signal 1: DAD1 A, Sig=330,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.490	BB	0.0518	1743.51855	498.75497	43.8009
2	3.753	BV	0.0548	2237.03442	596.36133	56.1991



5-cyanotryptophan (Table 4, entry 4). White solid (33 mg, 49% yield), corresponding to 2450 turnovers. ^1H NMR (500 MHz, D_2O) δ 7.92 (dd, J = 1.6, 0.7 Hz, 1H), 7.47 (dd, J = 8.5, 0.7 Hz, 1H), 7.39 (dd, J = 8.5, 1.5 Hz, 1H), 7.35 (s, 1H), 4.26 (dd, J = 7.0, 5.5 Hz, 1H), 3.37 (ABXY, J_{AX} = 7.0 Hz, J_{AY} = 0.7, J_{BX} = 5.5 Hz, J_{BY} = 0.8 Hz, J_{AB} = 15.4 Hz, ν_{AB} = 28.0 Hz, 2H). ^{13}C NMR (100 MHz, D_2O) δ 172.1, 138.3, 127.4, 126.2, 124.8, 124.3, 121.8, 112.7, 107.5, 100.7, 53.4, 25.6. HRMS (FAB) (m/z) for $[\text{M}+\text{H}]^+$ $\text{C}_{12}\text{H}_{12}\text{N}_3\text{O}_2$ requires 230.0929, observed 230.0928.

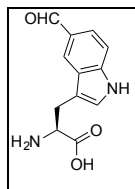


Signal 1: DAD1 A, Sig=330,8 Ref=off

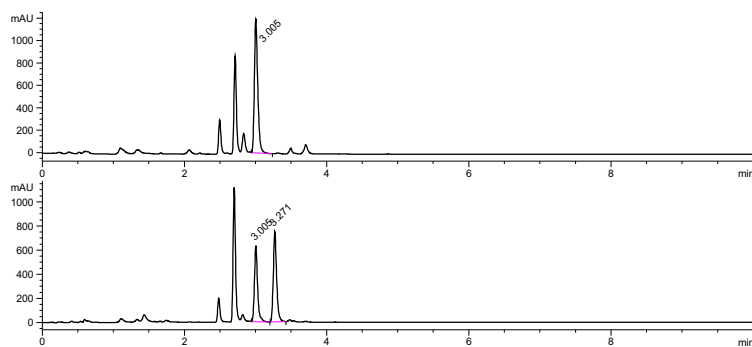
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.236	BV	0.0556	5448.84082	1426.40955	99.4750
2	3.566	VV	0.0422	28.75493	10.11823	0.5250

Signal 1: DAD1 A, Sig=330,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.270	BB	0.0512	2385.70728	691.76343	45.7334
2	3.539	BV	0.0541	2830.85156	765.56097	54.2666



5-formyltryptophan (Table 4, entry 5). Orange solid (22 mg, 32% yield), corresponding to 1600 turnovers. ^1H NMR (500 MHz, D_2O) δ 9.68 (d, J = 0.5 Hz, 1H), 7.98 (dd, J = 1.6, 0.7 Hz, 1H), 7.57 (dd, J = 8.6, 1.6 Hz, 1H), 7.43 (dt, J = 8.6, 0.6 Hz, 1H), 7.30 (s, 1H), 4.29 (dd, J = 6.9, 5.5 Hz, 1H), 3.37 (ABXY, J_{AX} = 6.9 Hz, J_{AY} = 0.7, J_{BX} = 5.5 Hz, J_{BY} = 0.7 Hz, J_{AB} = 15.6 Hz, ν_{AB} = 24.4 Hz, 2H). ^{13}C NMR (100 MHz, D_2O) δ 196.2, 172.1, 140.3, 128.0, 127.2, 126.4, 122.2, 112.5, 108.7, 53.4, 25.6. HRMS (FAB) (m/z) for $[\text{M}+\text{H}]^+$ $\text{C}_{12}\text{H}_{13}\text{N}_2\text{O}_3$ requires 233.0926, observed 233.0930.

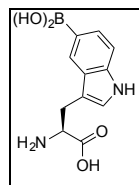


Signal 1: DAD1 A, Sig=330,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.005	BB	0.0484	3945.69775	1197.23047	100.0000

Signal 1: DAD1 A, Sig=330,4 Ref=off

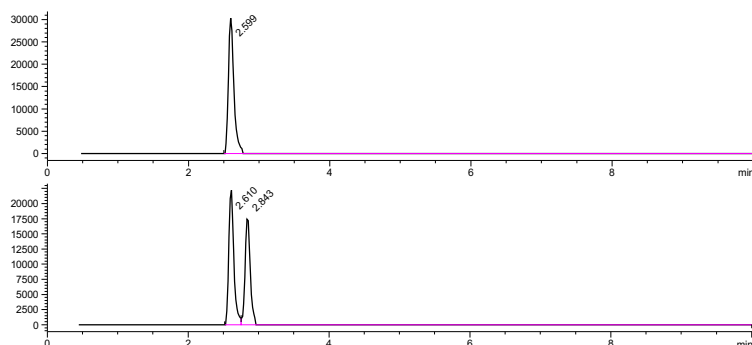
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.005	BB	0.0459	1932.20105	628.57422	44.4857
2	3.271	BB	0.0475	2411.21680	750.92450	55.5143



5-boronotryptophan (Table 4, entry 6). The substrate 5-boronoindole was subjected to the same procedure as entries 2–5, but with slight modifications. Due to competing proto-deborylation, the reaction mixture was not heated prior to the addition of enzyme. Furthermore, the product was sufficiently soluble in water that all extraction steps used neutral water instead of 0.1 M aq. HCl.

Finally, the higher polarity of the product, as well as the presence of tryptophan as a contaminant, hindered the chromatographic purification of the product. Thus, to better assess the yield, the product was partially purified chromatographically, as described above, then dissolved in 1000 μ L of D₂O that contained CH₃CN in a 104 μ M concentration. The solution was analyzed by ¹H NMR with a 10-s relaxation time, and the sum of the integrations of the signals at 3.93, 3.40, and 3.22 ppm was divided by the integration of the CH₃CN signal (2.03 ppm), indicating that the concentration of product was 38.4 μ M, which indicates a 38% yield and 1900 total turnovers.

¹H NMR (500 MHz, D₂O) δ 8.05 (t, J = 1.0 Hz, 1H), 7.55 (dd, J = 8.2, 1.2 Hz, 1H), 7.48 (dd, J = 8.3, 0.8 Hz, 1H), 7.26 (s, 1H), 3.93 (dd, J = 7.8, 5.0 Hz, 1H), 3.31 (ABX, J_{AX} = 7.8 Hz, J_{BX} = 5.0 Hz, J_{AB} = 15.2 Hz, ν_{AB} = 89.25 Hz, 2H). **¹³C NMR** (100 MHz, D₂O) δ 176.1, 160.7, 137.8, 126.9, 126.6, 125.1, 124.5, 111.4, 108.5, 55.3, 27.1. **HRMS** (ESI) (m/z) for [M+H]⁺ C₁₁H₁₄BN₂O₄ requires 249.1047, observed 249.1048.

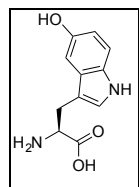


Signal 3: MSD1 483, EIC=482.7:483.7

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	2.599	BBA	0.0865	1.63070e5	3.03603e4	100.0000

Signal 3: MSD1 483, EIC=482.7:483.7

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
1	2.610	BV	0.0771	1.11103e5	2.23479e4	55.2562
2	2.843	VBA	0.0779	8.99656e4	1.78465e4	44.7438

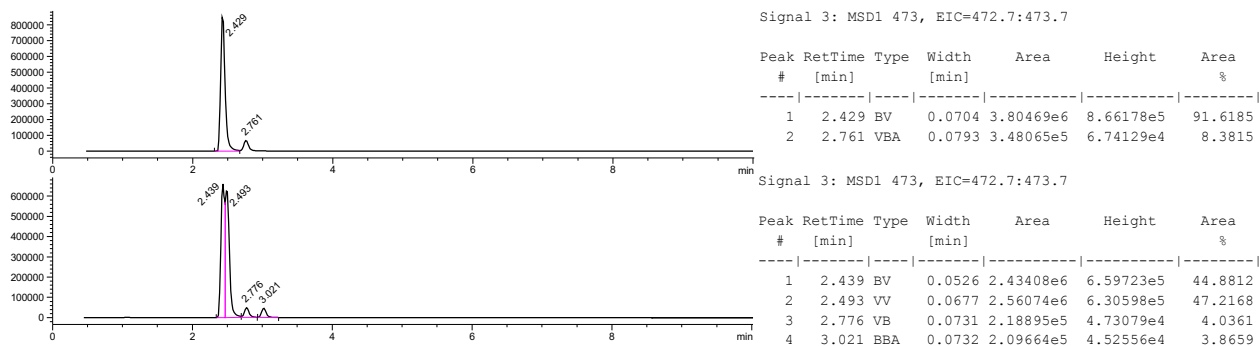


5-hydroxytryptophan (Table 4, entry 7). The reaction with this substrate followed the general procedure, but with modified post-reaction processing. The reaction was performed with 33.3 mg of 5-hydroxyindole (250 μ M), 1.2 equiv of L-Ser, and 0.01 mol % of enzyme. The starting materials were dissolved in 1250 μ L DMSO and 23.5 mL of potassium phosphate buffer, for a final reaction volume of about 25 mL. The reaction was heated for 2 hours, then cooled in ice. The reaction mixture was subjected to the same washing procedure described above, except that water was used in place of 0.1 M aq. HCl. Due to the higher polarity of the product, as well as the presence of a contaminant that is presumed to be an oxidative side-

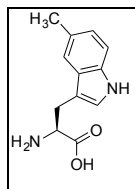
product, the product could not be purified chromatographically. As a result, the material was partially purified by chromatography as described above, then dissolved in 1000 μL of D_2O that contained CH_3CN in a 104 μM concentration. The solution was analyzed by ^1H NMR with a 10-s relaxation time, and the sum of the integrations of the signals at 3.73, 3.20, and 3.01 ppm was divided by the integration of the CH_3CN signal (1.98 ppm), indicating that the concentration of product was 233 μM , which indicates a 93% yield and 9300 total turnovers.

^1H NMR (500 MHz, D_2O) δ 7.26 (dd, $J = 8.7, 0.6$ Hz, 1H), 7.12 (s, 1H), 7.03 (dd, $J = 2.5, 0.6$ Hz, 1H), 6.75 (ddd, $J = 8.7, 2.4, 0.5$ Hz, 1H), 3.73 (dd, $J = 8.0, 4.8$ Hz, 1H), 3.11 (**ABXY**, $J_{\text{AX}} = 8.0$ Hz, $J_{\text{AY}} = 0.6$ Hz, $J_{\text{BX}} = 4.9$ Hz, $J_{\text{BY}} = 0.8$ Hz, $J_{\text{AB}} = 15.0$ Hz, $\nu_{\text{AB}} = 93.3$ Hz, 2H). **^{13}C NMR** (125 MHz, D_2O) δ 177.3, 148.9, 131.4, 127.6, 125.7, 112.7, 111.8, 107.9, 102.8, 55.4, 27.8. **HRMS** (FAB) (m/z) for $[\text{M}+\text{H}]^+$ $\text{C}_{11}\text{H}_{13}\text{N}_2\text{O}_3$ requires 221.0926, observed 221.0922.

* Derivatization with FDNP-alanamide gave two isomeric products, possibly due to competing arylation of the phenolic oxygen.

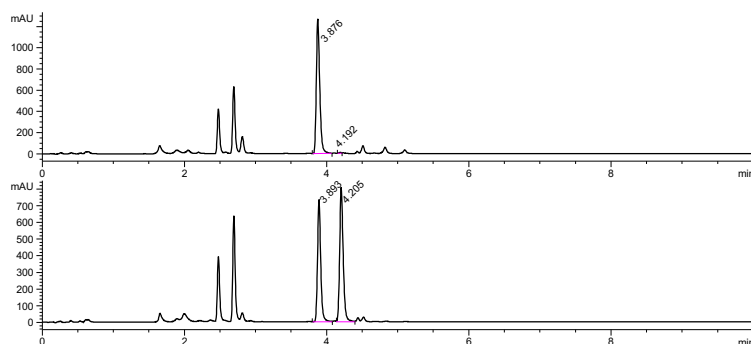


Modification for Table 4, entries 8–9. Initially, it was observed that the products of these reactions developed a red color during the post-reaction processing. This was accompanied by the appearance of a contaminant that possessed the expected mass of the product plus 16 mass units. As a result, the product solutions were subjected to one freeze-pump-thaw cycle prior to any concentration *in vacuo*. This appeared to suppress the decomposition.



5-methyltryptophan (Table 4, entry 8). The reaction was performed with 32.8 mg of 5-methylindole (250 μM), 1.2 equiv of L-Ser, and 0.01 mol % of enzyme. The starting materials were dissolved in 1250 μL DMSO and 23.5 mL of potassium phosphate buffer, for a final reaction volume of about 25 mL. The reaction was heated for 2 h, then processed as described above to afford 5-methyltryptophan hydrochloride as a white solid (58 mg, 91% yield), corresponding to 9100 turnovers.

^1H NMR (500 MHz, D_2O) δ 7.44 (s, 1H), 7.39 (d, $J = 8.3$ Hz, 1H), 7.24 (s, 1H), 7.09 (dd, $J = 8.4, 1.6$ Hz, 1H), 4.31 (dd, $J = 7.3, 5.2$ Hz, 1H), 3.40 (**ABX**, $J_{\text{AX}} = 7.4$ Hz, $J_{\text{BX}} = 5.3$ Hz, $J_{\text{AB}} = 15.4$ Hz, $\nu_{\text{AB}} = 47.4$ Hz, 2H), 2.40 (s, 3H). **^{13}C NMR** (125 MHz, D_2O) δ 172.1, 134.6, 129.3, 126.8, 125.5, 123.7, 117.6, 111.9, 105.9, 53.4, 25.9, 20.5. **HRMS** (FAB) (m/z) for $[\text{M}+\text{H}]^+$ $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_2$ requires 219.1134, observed 219.1132.

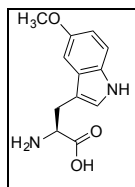


Signal 1: DAD1 A, Sig=330,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.876	VV	0.0484	4178.52832	1269.27234	99.4886
2	4.192	BV	0.0321	21.47815	9.97874	0.5114

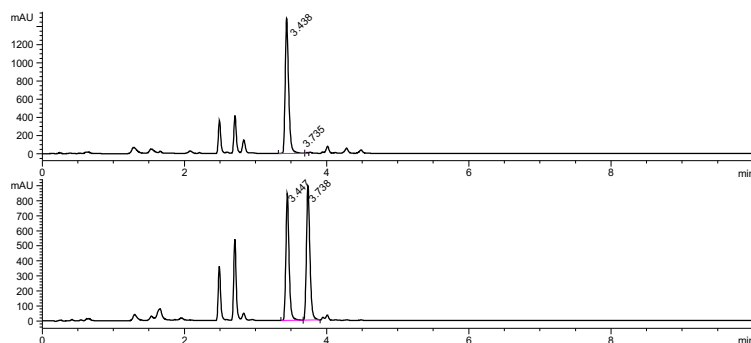
Signal 1: DAD1 A, Sig=330,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.893	BV	0.0458	2247.41968	733.06561	45.2738
2	4.205	VV	0.0492	2716.64355	808.07471	54.7262



5-methoxytryptophan (Table 4, entry 9). The reaction was performed with 73.6 mg of 5-methoxyindole (500 μ M), 1.2 equiv of L-Ser, and 0.01 mol % of enzyme. The starting materials were dissolved in 500 μ L DMSO and 8938 μ L of potassium phosphate buffer, for a final reaction volume of about 10 mL. The reaction was heated for 2 h, then processed as described above to afford 5-methoxytryptophan hydrochloride as a white solid (103 mg, 76% yield), corresponding to 7600 turnovers.

^1H NMR (500 MHz, D_2O) δ ^1H NMR (500 MHz, Deuterium Oxide) δ 7.37 (dd, J = 8.9, 0.5 Hz, 1H), 7.23 (s, 1H), 7.12 (dd, J = 2.5, 0.6 Hz, 1H), 6.86 (ddd, J = 8.9, 2.5, 0.4 Hz, 1H), 4.28 (dd, J = 7.1, 5.3 Hz, 1H), 3.35 (ABXY, J_{AX} = 7.2 Hz, J_{AY} = 0.7 Hz, J_{BX} = 5.4 Hz, J_{BY} = 0.8 Hz, J_{AB} = 15.5 Hz, ν_{AB} = 34.9 Hz, 2H). **^{13}C NMR** (100 MHz, D_2O) δ 172.1, 153.0, 131.7, 126.9, 126.2, 112.9, 111.9, 106.1, 100.5, 56.0, 53.3, 25.7. **HRMS** (FAB) (m/z) for $[\text{M}+\text{H}]^+$ $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_3$ requires 235.1083, observed 235.1084.



Signal 1: DAD1 A, Sig=330,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.438	BV	0.0492	4998.42236	1488.41821	99.5761
2	3.735	VV	0.0311	21.27838	10.29514	0.4239

Signal 1: DAD1 A, Sig=330,8 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.447	BB	0.0463	2631.31177	844.92169	47.0564
2	3.738	BB	0.0486	2960.51782	895.60193	52.9436

7. References

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8. ^1H and ^{13}C NMR spectra of tryptophan derivatives

